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Efficacy of a selective isolation procedure for members of the *Pseudallescheria boydii* complex

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Abstract Members of the *P. boydii* species complex (Microascaceae) are frequently involved in human opportunistic disease. Studies indicate that the prevalent habitat of *P. boydii sensu lato* is in agriculturally exploited or otherwise human-impacted soils. Quantitative analysis of fungal indicators in the environment can be exploited for monitoring of general environmental changes, as well as for understanding local population changes and its epidemiological consequences. In this study we present the development and testing of a semi-selective isolation procedure for *P. boydii* and related species. Three general media, DG18, rose bengal agar and five variations of modified Leonian's agar with and without benomyl were tested. Germination percentages of *P. boydii*, *S. prolificans*, *Petriella* spp. and *Aspergillus fumigatus* (control) were evaluated. Tests were carried out on the success of *P. boydii* isolation from inoculum mixed with *A. fumigatus*. Subsequently the procedure was applied to water, sediment and soil samples. On the newly introduced semi-selective medium (SceSel+), the germination of *P. boydii* was superior or similar to that seen on the other media tested. *P. boydii* was isolated from mixed cultures only on SceSel+ but not on SceSel without benomyl. Isolation from environmental sources with SceSel+ was successful, and human impacted soil was confirmed as the predominant habitat of *P. boydii*.

Keywords Pseudallescheria · Isolation · Selective medium · SceSel+ · Ecologic niche

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Introduction

Members of the *Pseudallescheria boydii* complex (*P. boydii* (Shear) McGinnis et al. and *S. apiospermum*) and *S. prolificans* are members of the Microascaceae, which are frequently involved in human opportunistic disease. Infections occur in immunocompetent patients and can be subcutaneous or deep, such as in the case of mycetoma and arthritis. Also subclinical colonization of the respiratory tract including lungs of patients with cystic fibrosis is common (Cimon et al. 2000). In the immunocompromised patient, systemic infections occur, which often involve the central nervous system (Gueho and de Hoog 1999). A unique clinical entity is brain infection after aspiration of contaminated water after

accidents. In one such case, isolation of *P. boydii* was possible at the site of an accident (Buzina et al. 2006).

Studies so far, seem to indicate that agriculturally heavily exploited or otherwise human impacted environments like cities and industrial areas are main components of the ecological niche of *P. boydii* (Guarro et al. 2006; Kaltseis and Rainer 2006). *Pseudallescheria boydii*, is known to be fit for growth in such environments by virtue of its ability to degrade alkanes (April et al. 1998) and to grow in microaerophilic environments (de Hoog et al. 1994). Regardless of the ecological tendencies mentioned above, the entire niche and the in situ biomass levels of *P. boydii* are not known accurately enough to allow infection risk assessment (Guarro et al. 2006). In addition, we lack understanding of the role of *P. boydii* as a decomposer, and we are not fully exploiting its potential to serve as indicator organism of environmental changes. Selective isolation may comprise a useful tool to generate data for clinical and ecological purposes. Applications may be designed for diagnosis of mycoses, tracing of routes of infection, detection and monitoring of high-risk environments, and, in the laboratory, determination of the minimal load of fungal propagules needed to cause infection in model organisms.

Quantitative analysis is crucial in the ecological investigation of fungal populations. Changes in microbial communities, such as the loss or gain of certain species, may reflect general environmental changes or changing local influences. In addition, shifts in the population structure may help to monitor the impact of anthropogenic activities on the environment. Studies on the diversity and abundance of soilborne fungi are generally based on culture techniques. Use of such methods has disadvantages. Culture media and growth conditions strongly influence which organisms will germinate and manifest sufficiently vigorous growth to prevail against competitors in the artificial situation. Culture techniques alone therefore do not allow accurate determination of fungal biodiversity in environmental samples. It remains difficult to obtain a realistic picture of the ecological role of particular fungi just from recording "colony forming units" (CFU) in various situations. Another limitation intrinsic to culture methods is the possible over-representation of fast growing strains on the complex isolation media that are mostly used. Selective media have therefore been designed to overcome this problem. One of the advantages of selective isolation is that it allows a reasonable approximation of target organism quantities.

In this study we present the development of a semi-selective isolation procedure for the medically relevant fungi of the genera *Pseudallescheria* and *Scedosporium*.

Material and methods

Media preparation

Medium variants tested (Table 1) included several media widely used in soil isolations. Some of these, such as Martin's Rose Bengal agar or dichloran glycerol agar (DG18), have selective properties or restrict colony diameters of fast-growing fungi (Samson et al. 2004). A benomyl-containing medium was also designed based on the addition of this selective compound to a medium promoting growth and sporulation of a wide range of fungi (modified Leonian's agar, MLA) (Summerbell 1993). The MLA-benomyl medium supplemented with antibiotics (ciprofloxacin, chloramphenicol, streptomycin, and dichloran) was denoted SceSel+ (Table 1). Before any environmental testing was performed, ten media and variants were examined by comparing the germination percentage of ascospores and conidia achieved on each of them at 37°C. Target organisms, including strains of *P. boydii*, *Pseudallescheria angusta*, *P. ellipsoidea*, and *S. apiospermum* (referred to as *P. boydii* complex in Fig. 1) two *S. prolificans*, and one each of *Petriella guttulata* and *P. setifera*, as well as two *Aspergillus fumigatus* strains representing contaminant organisms (Table 2).

Germination percentage in pure cultures

A sterile cotton swab was used to harvest conidia and, where formed, ascospores from cultures of the isolates listed in Table 2. These propagules were suspended in a Tween 80 0.01%/NaCl 0.85% solution in de-ionized water (w/v). Inoculum counts were determined microscopically with a Thoma chamber

Table 1 Composition of the applied selective and general media

Scedosporium selective Agar (SceSel+)	Malt extract, 6.25 g; maltose, 6.25 g; mono-potassium-phosphate, 1.25 g; yeast extract, 1.0 g; magnesium sulfate · 7 H_2O, 0.625 g; soy peptone, 0.625 g; chloramphenicol, 0.1 g[a]; ciprofloxacin, 0.1 g[b]; streptomycin sulfate, 0.1 g[c]; dichloran, 2 mg[d]; benomyl, 6 mg[e]; agar, 20.0 g; a. d., 983 ml.
SceSel–	SceSel+ without benomyl.
MLBCS + D	SceSel+ without ciprofloxacin, benomyl suspended in sterile water.
MLBCS + B in methanol	SceSel+ without ciprofloxacin and dichloran, benomyl dissolved in methanol.
MLA + $B_{susp.}$	SceSel+ without chloramphenicol, streptomycin sulfate, ciprofloxacin, and dichloran; benomyl suspended in sterile water.
MEA 2%	Malt extract, 20.0 g; glucose, 20.0 g; peptone from soy, 1.0 g; agar, 20.0 g; a. d., 1 l.
Rose bengal	Agar, 15.0 g; peptone from soy, 5.0 g; glucose, 10.0 g; mono-potassium-phosphate, 1.0 g, magnesium-sulfate · 7 H_2 O, 0.5 g; dichloran, 2 mg; rose bengal, 25 mg, benomyl, 8 mg; a. d., 1 l.
DG18	Peptone, 5.0 g; glucose, 10 g; mono-potassium-phosphate, 1.0 g; magnesium-sulfate · 7 H_2O, 0.5 g; dichloran, 2 mg; glycerol, 220.0 g; chloramphenicol, 0.1 g; agar, 15.0 g; a. d., 1 l.
PDA	Merck 10130, commercial PDA medium.
SGA	Peptone, 10.0 g; glucose, 10.0 g; agar, 15 g; a. d., 1 l.

[a] Dilute in 5 ml ethanol 96%; [b]dilute in 1 ml 1 M NaOH and add to the 55°C agar; [c]dilute in 5 ml a. d. filter sterilize and add to the 55°C agar; [d]One milliliter of a 0.2% solution in ethanol; [e]dilute in 5 ml methanol, add to the 55°C agar

Table 2 Strains

Species	Collection No.	Source of isolation
Pseudallescheria boydii	CBS 101.22 T	Mycetoma, USA (Texas)
P. boydii	CBS 101721	Mud of ditch, the Netherlands ('s Graveland)
P. boydii	CBS 101726	Mud of urban pond, the Netherlands (Hilversum)
P. boydii	CBS 117411	Garden soil, Spain (Barcelona)
P. Boydii	CBS 117426	Sputum of CF-patient, France (Giens)
P. angusta	CBS 108.54	Soil, Congo
P. ellipsoidea	CBS 418.73 T	Soil, Tajikistan
Scedosporium apiospermum	CBS 987.73	Otitis, Czech Republic (Brno)
S. prolificans	CBS 100390	Blood of leukemic (AML) patient, Germany (Frankfurt)
S. prolificans	CBS 114.90 T	Bone biopsy of 6-year-old male, USA
Petriella guttulata	CBS 362.61	Partridge dung, Germany
Petriella setifera	CBS 559.80	Woodland soil, Jamaica (Ochos Rios)
Aspergillus fumigatus	CBS 192.65	Animal feed, the Netherlands
A. fumigatus	JR 110	Lung of a haematology patient, Austria (Innsbruck)
A. fumigatus	JR 192A	Air of a haematology unit, Austria (Innsbruck)

CBS = Collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; JR = working collection of the Institute of Microbiology, LFU-Innsbruck, Austria; T = ex-type strain

(0.1 mm × 0.0025 mm^2). The suspensions were serially diluted in a series ranging from 10^6 to 10^1 conidia or spores per ml. The dilutions containing 10^1, 10^2, and 10^3 propagules/ml were inoculated on selective and non-selective solid media (Table 2) in triplicate for each dilution. Colonies were counted after incubation at 37°C for 5–7 days on plates where reliably countable numbers of colonies (i.e., 5–50) developed. The mean germination percentage was calculated based on

data from the three parallel trials per isolate (Table 3).

Germination in mixed cultures

Two dilutions of an *A. fumigatus* conidial suspension, 3×10^3 and 3×10^2 conidia/ml, were plated in combination with two suspensions of *P. boydii* (1.6×10^6 and 1.6×10^4 conidia/ml) on SceSel+ and SceSel–. Each plate was inoculated with 0.2 ml *A. fumigatus* and 0.2 ml *P. boydii* suspension resulting in 600 and 60 conidia/plate together with 3.2×10^5 and 3.2×10^3 conidia per plate. Colonies were counted after incubation at 36°C for 4–5 days. The temperature was changed from 37°C to 36°C for technical reasons during this experiment.

Isolation of *P. boydii* from soil and sediment samples

To concretize the niche of *P. boydii* and to test the potential performance of SceSel+ medium in a larger ecological study, we assayed the abundance of *P. boydii* in water, sediment, and soil samples from a single locality. The values (Fig. 2) are illustrated as boxplots calculated from ten replicate plates. The samples were taken from the outflow of a pond frequented by waterfowl in a public park in Innsbruck, Austria. About 5–15 g sediment or soil was collected in sterile 50 ml plastic tubes. Materials were diluted 3.3×10^{-1} (w/v) in Tween 80 0.01%/NaCl 0.85%. Fungal propagules were extracted by shaking the suspension (end-over-end) for 1 h. A total of 250 µl of the suspension were inoculated on

Table 3 Mean values of germination from three parallels given in percent of the propagule counts determined by microscopic means

	MLBCS	MLBCS + D	MLBCS + B in Meth	MLA + B_{susp}	SceSel+	Rose bengal	MEA	DG18	PDA	SGA
P. boydii										
CBS 101.22	24.2	23.3	27.0	34.9	40.4	24.2	25.7	15.8	37.6	26.0
CBS 101721	31.2	31.6	26.3	30.1	42.3	12.4	27.1	0	19.2	22.2
CBS 101726	22.8	19.3	21.0	20.2	33.7	19.2	16.6	0	17.8	9.5
P. angusta										
CBS 108.54	19.0	18.5	19.7	17.6	22.4	20.0	19.2	0	16.7	16.7
P. ellipsoidea										
CBS 418.73	66.2	64.2	59.7	41.5	83.9	75.8	61.5	0	56.3	70.4
S. apiospermum										
CBS 987.73	33.8	29.7	34.8	26.1	44.0	28.8	30.2	0	21.4	23.4
S. prolificans										
CBS 100390	30.0	34.0	33.0	19.4	49.0	37.0	44.2	26.4	23.9	16.8
CBS 114.90	54.5	56.0	72.2	11.1	30.3	50.5	56.3	92.6	61.8	55.2
Pe. guttulata										
CBS 362.61	0	0	0	0	0	0	6.7	0	16.4	42.7
Pe. setifera										
CBS 559.80	0	0	0	99.6	0	–	92.0	48.4	84.0	92.0
A. fumigatus										
JR 110	0	0	0	0	0	0	100.0	100.0	100.0	85.4
JR 192A	0	0	0	0	0	0	100.0	91.1	100.0	97.4

SceSel+ on ten replicate plates. After 5–7 days incubation at 37°C, colony counts were obtained; outgrowing fungi were identified based on their colonial and microscopic features (de Hoog et al. 2000).

Isolation of *P. boydii* from polluted water samples

Water samples were plated directly on ten replicate plates of SceSel+, at 0.5 ml per plate. After 1 week of incubation at 37°C, colonies were counted and identified as described above. The median, minimum, maximum and quartiles of CFU value/ml water were calculated.

Results

Germination percentage in pure culture

The average germination percentage of *Pseudallescheria* and *S. apiospermum* propagules on SceSel+ was 44.4% and ranged from 22.4% (CBS 108.54) to 83.9% (CBS 418.73). Average germination was somewhat lower on other media based on MLA and lower on rose bengal agar (28.4% MLA + $B_{susp.}$, 32.9% MLBCS; 30.1% rose Bengal, Table 3). On non-selective media, the mean germination levels of *P. boydii* and *S. apiospermum* strains lay between 9.5% and 70.4%. Only one strain of *P. boydii* (CBS 101.22) was found to germinate on DG18 (15.8%).

The mean germination of *Scedosporium prolificans* conidia ranged from 15.3% on MLA + $B_{susp.}$ to 59.5% on DG18. On SceSel+, germination levels of the two tested isolates were 49.0% and 30.3%, values that fall within the range seen for the other benomyl-containing media, except for MLA + $B_{susp.}$, for which a lower mean level was calculated. On the non-selective media PDA, SGA, and MEA germination levels were found to fall between 36.0% (SGA) and 50.3% (MEA).

Petriella species either did not grow at 37°C or were found to form much smaller colonies than *P. boydii* or *S. prolificans* within 5–7 days at 37°C on all tested media. *Aspergillus fumigatus* did not germinate on any of the benomyl-containing media, although a germination level of 91.4–100% was observed on non-selective media.

Germination in mixed cultures: comparison of SceSel with and without benomyl

Inoculating *A. fumigatus* and *P. boydii* together on SceSel– in concentrations of 3×10^2 to 1.6×10^6 resulted in a large number of small *P. boydii* colonies observable (1–2 mm diameter) between large, sporulating *A. fumigatus* colonies. On the SceSel+ plates only *P. boydii* was detectable (no growth of

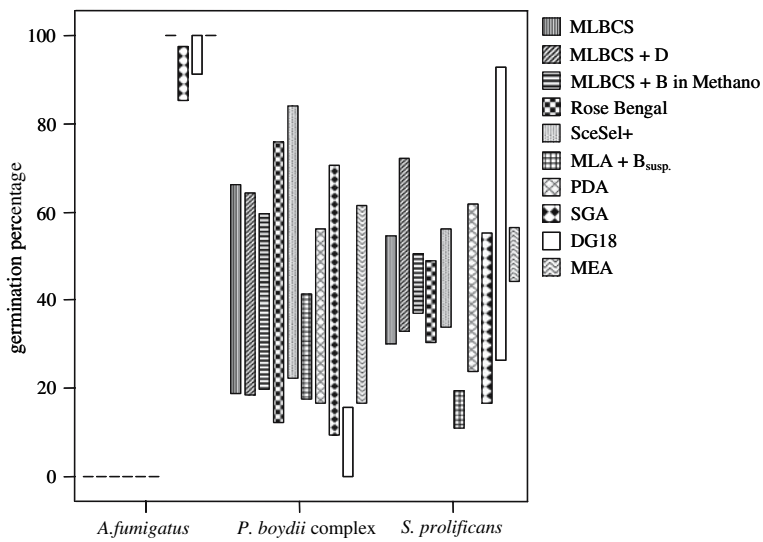

Fig. 1 Minimum and maximum germination levels of *P. boydii* complex, *S. prolificans* and *A. fumigatus* ascospores/conidia on general and selective media given as percentage of the microscopically estimated counts. Incubation was at 37°C for 5–7 days

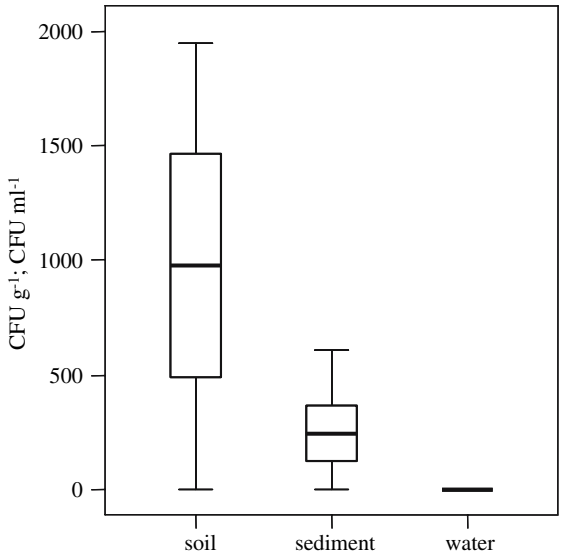

Fig. 2 Boxplot of CFU/g dry weight (soil, sediment) and CFU/ml (water) isolated from samples from an urban pond

A. fumigatus) (Table 4). The detection limit of *P. boydii* in the presence of *A. fumigatus* on SceSel+ was found to lie below 1.6×10^3 conidia/ml. When *A. fumigatus* was not suppressed by benomyl, *P. boydii* was either not detectable or was too extensively overgrown to be enumerated. The germination percentage of *P. boydii* ascospores/conidia in the presence of *A. fumigatus* on SceSel+ was lower than in pure culture (Tables 3, 4).

Application of SceSel+ for the detection of *P. boydii* in soil, sediment, and water

The median value of CFU counts of *P. boydii* were 975.6 CFU/g dry weight (d.w.) in soil and 242 CFU/g d.w. in sediment. The fungus was not detected in water on this occasion (Fig. 2). In earlier experimental samplings (data not shown) the water of this pond was found to contain low CFU numbers of *P. boydii*.

Discussion

In comparisons of various general and selective media, it was shown that benomyl-amended MLA media do not exert negative effects on germination levels of *Pseudallescheria* and *Scedosporium* strains (Table 3, Fig. 1). Similarly, antibacterial and antimycotic agents had no adverse effect on the germination of *P. boydii* and *S. prolificans* at 37°C. Diluting benomyl in methanol instead of suspending it directly in the 55°C agar was introduced successfully and provides improved dispersal of this component. Other Microascaceae that might be isolated from soil or wood, e.g., *Petriella* spp., did not grow on SceSel+, or grew much slower at 37°C than at room temperature. Whereas a high proportion of *A. fumigatus* conidia germinated on media without benomyl, only a small proportion of *P. boydii* ascospores and conidia did so. Likewise, in mixed cultures of *P. boydii* and *A. fumigatus*, a more than 50-fold higher inoculum level of *P. boydii* led to CFU counts differing from *A. fumigatus* by a factor of only 2.3.

No growth of *P. boydii* was found on DG18; we therefore tested dichloran separately on MLBCS + D. In this test, germination levels were similar to those observed on other MLA-based media. The factor causing the inhibition of *P. boydii* on DG18 obviously is the very low water activity of this medium and not the presence of dichloran. To reduce the chance that SceSel+ would be overgrown by benomyl-tolerant Zygomycetes, dichloran was added to the medium. Because *S. prolificans* differed from *P. boydii* by growing on DG18, this medium can be used supplementary to microscopy for rapid non-molecular species distinction.

Table 4 Outgrowth from different mixtures of *A. fumigatus* and *P. boydii* on SceSel– and SceSel+, in CFU/ml

	A		B		C		D	
	SceSel–	SceSel+	SceSel–	SceSel+	SceSel–	SceSel+	SceSel–	SceSel+
A. fumigatus	75	0	ov.	0	75	0	ov.	0
P. boydii	ov.	ov.	ov.	ov.	175	125	nd.	125

A—*A. fumigatus* 300 conidia/ml, *P. boydii* 1.6×10^6 conidia/ml; B—*A. fumigatus* 3×10^3 conidia/ml, *P. boydii* 1.6×10^6 conidia/ml; C—*A. fumigatus* 300 conidia/ml, *P. boydii* 1.6×10^4 conidia/ml; D—*A. fumigatus* 3×10^3 conidia/ml, *P. boydii* 1.6×10^4 conidia/ml; nd = not detectable; ov. = overgrown

In addition to the problem of overgrowth by fast growing fungi, fungal soil isolation studies are complicated by problems posed by soil bacteria, especially *Pseudomonas* species. In the present study, it was shown that various antibiotic mixtures did not affect the germination of *P. boydii* ascospores/conidia but decreased bacterial growth in cultures of all sample types.

In experiments with artificially mixed inocula, it was demonstrated that the selective effect of SceSel+ is high, and that other thermotolerant fungi like *A. fumigatus* did not overgrow *P. boydii* on this medium. Therefore, underestimation of *P. boydii* in environmental samples is minimized when SceSel+ is used for isolation.

During field sampling, strains of *P. boydii*, some yeasts, and highly resistant bacteria were isolated. These isolates were not identified. Summerbell et al. (1993) stated, that several genera of fungi are resistant to benomyl, among them common ascomycetous and basidiomycetous yeasts (*Candida*, *Cryptococcus*, *Hansenula*, *Kloeckera*, *Malassezia*, *Pichia*, *Rhodotorula*, *Saccharomyces*, *Trichosporon*), members of the Pleosporaceae as well as *Geotrichum candidum* and Microascaceae. In our experience, these fungi do not represent a practical problem during isolation of *P. boydii*, because it grows in conditions where most competing organisms are inhibited by antibiotics or temperature. A minor problem was met with certain Zygomycetes which were resistant to the added antimycotics. As their growth was inhibited to a large extent, enumeration of *P. boydii* was always possible. Such overgrowth makes subculturing and microscopic investigation difficult, and therefore complicates species identification of members of the *Pseudallescheria/Scedosporium* clade.

Several publications (Pinto et al. 2001) have suggested that the preferred habitat of *P. boydii* is mainly in polluted water. Rainer and de Hoog (2006) postulated that ascomata of *Pseudallescheria* were adapted to release the ascospores in moist environments. Our application of ScelSel+ allowed assigning the main habitat of *P. boydii* to soil. But, this does not exclude the dispersal of ascospores by water: high *P. boydii* counts in soil may lead to an accumulation in the sediment through carriage by water.

One of the main types of events giving rise to *Scedosporium* infection is a near-drowning incident followed by coma (Guarro et al. 2006). Apparently, a mechanical disturbance of the sediment layers is necessary to suspend infectious particles into the water, which is subsequently aspirated. Probably this circumstantial infection route is the reason why infections with *P. boydii* are relatively uncommon.

Detection of *P. boydii* in the environment is relevant because this fungus is able to mineralize hydrocarbons and because of its clinical significance (Guarro et al. 2006). Our data demonstrate that non-selective isolation procedures underestimate the in situ frequency of *Pseudallescheria* and *Scedosporium*. Therefore, methods similar to the ones introduced here are crucial to studies on ecological reservoirs and populations of *P. boydii*. They facilitate risk assessment of the potential *P. boydii* habitats that are ever more abundant as a result of human activities.

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